Esrrb Activates *Oct4* Transcription and Sustains Self-renewal and Pluripotency in Embryonic Stem Cells*

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The genetic program of embryonic stem (ES) cells is orchestrated by a core of transcription factors that has OCT4, SOX2, and NANOG as master regulators. Protein levels of these core factors are tightly controlled by autoregulatory and feed-forward transcriptional mechanisms in order to prevent early differentiation. Recent studies have shown that knockdown of *Esrrb* (estrogen-related-receptor β), a member of the nuclear orphan receptor family, induces differentiation of mouse ES cells cultured in the presence of leukemia inhibitory factor. It was however not known how knocking down *Esrrb* exerts this effect. Herein we have identified two ESRRB binding sites in the proximal 5′-untranslated region of the mouse *Oct4* gene, one of which is in close proximity to a NANOG binding site. Both ESRRB and NANOG are necessary for maintaining the activity of this promoter in ES cell lines. We have also demonstrated that the two transcription factors interact through their DNA binding domains. This interaction reciprocally modulates their transcriptional activities and may be important to fine-tune ES cell pluripotency. Supporting all of these data, stable transfection of *Esrrb* in ES cell lines proved sufficient to sustain their characteristics in the absence of leukemia-inhibitory factor. In summary, our experiments help to understand how Esrrb coordinates with *Nanog* and *Oct4* to activate the internal machinery of ES cells.

Two defining properties of ES[3] cells are the capacity to generate all cell types of an organism (pluripotency) and the ability to remain in a proliferative undifferentiated state (self-renewal). Despite the existence of differences, both human and mouse ES cells obtained from the inner mass of blastocysts can be cultured for prolonged periods of time without losing these two characteristics (1–3). Remarkably, the successful reprogramming of somatic cells to an embryonic-like state by nuclear transfer or less efficiently by fusion with ES cells demonstrated that specific factors exist in these cells that not only maintain but also can induce pluripotency (4–7). Understanding the molecular principles governing ES cell pluripotency is of outstanding interest for clinical purposes, as for example it may allow faithful differentiation into specific lineages or tissues. Recent large scale studies involving DNA microarray analysis upon forced differentiation of ES cells, chromatin immunoprecipitation (ChIP)-on-chip analysis, RNA interference, and proteomics approaches, have proven very powerful to gain insight into the internal machinery of ES cells (8–10). These and other reports have shaped an interlaced transcriptional network orchestrated by three transcription factors: OCT4, SOX2, and NANOG (11). A substantial proportion of the genes targeted by the three factors are transcription factors as well, making understandable how three proteins alone can control the complex ES cell behavior. Remarkably, the levels of these core factors must be tightly coordinated to prevent early differentiation. For example, repression or inactivation of *Oct4* differentiates ES cells along the trophoectodermal lineage, whereas high levels of *Oct4* can also cause differentiation mainly into primitive endoderm-like derivatives (12). OCT4, SOX2, and NANOG frequently co-occupy the promoters of their target genes and also bind to their respective own promoters to modulate their own expression. In addition, all three factors can bind to and activate the *Oct4* promoter (11).

As the amount of knowledge has increased, new constituents have been added to the ES cell pluripotency network, among which are *Tcf3*, *Rest*, and *Esrrb* (8, 9). *Tcf3* and *Rest* act at least in part by controlling the transcription levels of the main core factors (13, 14).

ESRRB and the related ESRRα and -γ share significant homology with the estrogen receptor and belong to the superfamily of nuclear receptors (15). They are classified as orphan receptors because they bind to DNA and are transcriptionally active in the absence of identified exogenous ligand (16). ESRRB can be co-immunoprecipitated as part of the protein complex associated with NANOG in ES cells (10), but the functional significance of this interaction was not known. In addi-

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[3] The abbreviations used are: ES, embryonic stem; AP, alkaline phosphatase; PIPES, 1,4-piperazinediethanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; LIF, leukemia-inhibitory factor; ChIP, chromatin immunoprecipitation.

tion, *Esrrb* knockdown using either short hairpin RNA lentiviruses or small interfering RNA oligonucleotides induces ES cell differentiation (8, 9). Possibly related to this, *Esrrb* is essential for adequate placental development, with *Esrrb* null mutant mice displaying abnormal trophoblast proliferation and precocious differentiation toward the giant cell lineage (17). In this report, we have investigated how *Esrrb* relates to the fundamental ES cell regulators *Oct4* and *Nanog* and have also demonstrated that forced expression of *Esrrb* alone is enough to maintain ES cell characteristics.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Media, and Primary Antibodies—HEK293T cells, mouse ES cell lines CGR8 and E14T, and mouse embryonic carcinoma cell lines P19 and F9 were cultured in Dulbecco's modified Eagle's medium high glucose (Invitrogen) with 10% (HEK293T), 15% (P19 and F9), or 20% (CGR8 and E14T) of fetal bovine serum (Hyclone) and penicillin/streptomycin. Medium for ES cells also contained LIF (1000 units/ml; Chemicon), nonessential amino acids (100 mM; Invitrogen), 0.55 mM 2-mercaptoethanol (Invitrogen), 1 mM pyruvate sodium (Invitrogen), and 2 mM GlutaMAX (Invitrogen). Stable transfectants were prepared by transient transfection with pPyCAGIP containing *Esrrb* or *Nanog* and selection with puromycin (Fluka). Alkaline phosphatase (AP) staining was performed using a kit from Chemicon. Antibodies used for this study were anti-NANOG and anti-ESRRB (made by us), anti-FLAG (Sigma), anti-Myc (Abcam), and anti-OCT4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Plasmids and Transfection Procedures—pPyCAGIP plasmids containing mouse NANOG full-length or deletions N1 (amino acids 1–197), N3 (amino acids 1–247), and N4 (amino acids 1–197/248–305), and FG-CD (amino acids 156–305) and NHWVP16 and NHVP16 plasmids have been described before (18–20). N5 and N6 were prepared by inserting PCR products encoding amino acids 1–95 (N5) of NANOG or 96–156 (N6) into a modified pCR3.1 II with green fluorescent protein and a Myc tag in the NH_2-terminal edge. pPyCAGIP plasmids containing full-length mouse ESRRB and deletions E1 (amino acids 1–211), E2 (amino acids 1–168), E3 (amino acids 169–433), E4 (amino acids 212–433), and E5 (amino acids 93–433) were generated by inserting PCR products into a modified pPyCAGIP plasmid containing an amino-terminal FLAG or Myc tag. NANOG luciferase reporter plasmid p5N and the luciferase reporter plasmid containing the *Oct4* promoter have been described before (18, 21); the pS2-luciferase plasmid was a kind gift from Dr. Vincent Giguere (McGill University Health Center, Montreal, Canada) (22). Mutagenesis of the *Oct4* promoter was performed using Pyrobest DNA polymerase (Takara) and DpnI (New England Biolabs) digestion. Primers were *muNanog-F* (5′-gccatcctggcccatGACCAggttgagtacttgtttagg), *muNanog-R* (5′-cctaaacaagtactcaaccTGGTCatgggccaggatggc), *muEsrrb-P1-F* (5′-ggattgtccagccaaTTccattgtcctgccccttccc), *muEsrrb-P1-R* (5′-gggaaggggcaggacaatggAAttggctggacaatcc), *muEsrrb-P1-F* (5′-gaattatcaggagttcaaTTCcagcttgttttatag), *muEsrrb-P2-R* (5′-ctataaaacaagctgGAAttgaactcctgataattc).

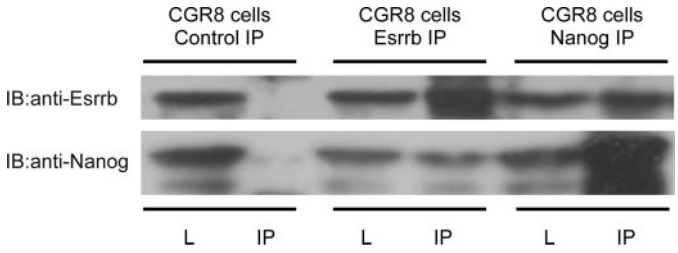

FIGURE 1. **Endogenous NANOG and ESRRB interact in ES cells.** Lysates from untransfected mouse CGR8 ES cells were immunoprecipitated with antibodies against NANOG or ESRRB; rabbit preimmune serum was used as a control. Immunoprecipitates (*IP*) or a percentage (5%) of the total lysate (*L*) were blotted for NANOG and ESRRB. One representative experiment of three is shown.

All newly prepared plasmids were verified by sequencing. Lipofectamine 2000 (Invitrogen) was used for all transfections. Cells were harvested 24 h after transfection for Western blotting and luciferase activity measurement and after 48 h for immunoprecipitation assays. Luciferase activity was measured using the dual luciferase reporter assay system from Promega. Transfections were carried out as duplicates and repeated at least three times. The indicated luciferase reporter genes (0.2 μg) and either pPyCAGIP vector (control) or the indicated expression vectors (0.5 μg) were used; pCMV-Renilla (0.005 μg) was used for normalization.

Western Blotting and Immunoprecipitation—Cells were lysed in radioimmune precipitation buffer with protease inhibitor mixture (Sigma) and phenylmethylsulfonyl fluoride (Amersco). Membranes were developed using AP with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate or ECL. For immunoprecipitation, cells were transfected in 60-mm dishes and lysed on ice in 400 μl of TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) with protease inhibitor mixture and phenylmethylsulfonyl fluoride. We used anti-FLAG-conjugated agarose beads from Sigma or anti-ESRRB and anti-NANOG combined with protein A/G-Sepharose beads (Sigma); 50 μl of the lysate supernatant was saved for the input control.

ChIP—DNA-protein complexes were cross-linked by adding 1% formaldehyde to cells cultured in 10-cm dishes, cross-linking was stopped with glycine. Cells were lysed on ice using a buffer containing 5 mM PIPES, pH 8.1, 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitor mixture. Nuclei were recovered by centrifugation and extracted with nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS with protease inhibitors and phenylmethylsulfonyl fluoride) on ice. Chromatin samples were sonicated on ice and centrifuged, and supernatants were precleared with protein A-Sepharose beads pretreated with salmon sperm (Sigma). Corresponding antibodies were incubated overnight with the samples in rotation at 4 °C; rabbit preimmune serum was used as control antibody. Fresh protein A-Sepharose beads with salmon sperm were added afterward, incubated for 2 h, and washed in rotation at room temperature sequentially with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM

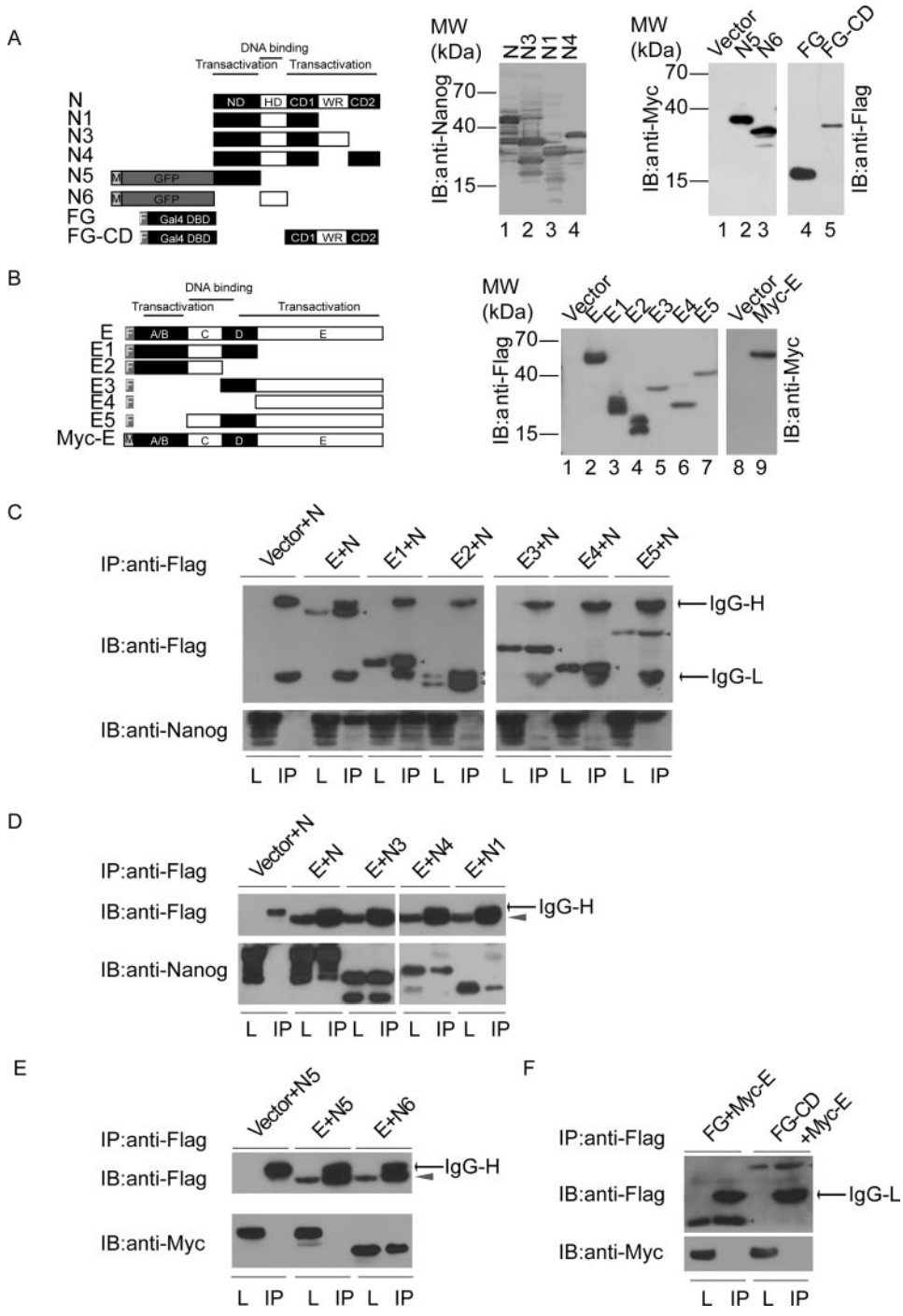

FIGURE 2. **NANOG and ESRRB interact through their DNA binding domains.** *A*, the scheme shows the respective protein domains contained in mouse NANOG deletions N1, N3, N4, N5, N6, FG, and FG-CD. *ND*, amino-terminal transactivation domain; *HD*, homeodomain and corresponds to the DNA binding domain of NANOG. The carboxyl-terminal transactivation domain of NANOG is composed of the CD1, WR, and CD2 subdomains. *Panels* on the *right-hand side* show Western blots with anti-NANOG (*left*), anti-Myc (*middle*), or anti-FLAG (*right*) of lysates from transiently transfected HEK293T cells and demonstrate adequate expression and sizes of these constructs. One representative experiment is shown. *B*, the scheme depicts mouse ESRRB and the domains contained in deletions E1–E5; the DNA binding domain of ESRRB contains the entire C domain and also a part of D. On the *right-hand side*, Western blots using anti-FLAG (*left*) or anti-Myc (*right*) antibodies demonstrate adequate expression and sizes of these constructs in transiently transfected HEK293T cells. One representative experiment is shown. *C–F*, immunoprecipitation (*IP*) with the indicated antibodies of HEK293T cells transiently transfected with the indicated plasmids. A fraction (5%) of the total lysate (*Input*) was used as a control. *Arrows* (*black*) are used to indicate heavy and light immunoglobulin chains and, where convenient, also (in *red*) NANOG and ESRRB deletions. One representative experiment of four is shown.

EDTA, 10 mM Tris-HCl, pH 8.0), and finally two times in TE buffer. Chromatin was eluted with 500 μl of elution buffer (50 mM $NaHCO_3$, 1% SDS) at room temperature. RNase A (Ameresco) and NaCl were added to the eluates, and samples were incubated at 65 °C for 4–5 h to reverse the cross-linking. DNA was precipitated with ethanol. The following primers were used for PCR amplification: P1 forward (5′-ggggattggggctcaggaggggggt), P1 reverse (5′-tctccctcccccacctcctcatc), P2 forward (5′-atgtgctatgtgtagctgtgtgta), P2 reverse (5′-tagccaggacagagttctgagcct), control forward (5′-gatggaatactgtgctctgaaaac), and control reverse (5′-catcctctggagagcctaaaacat).

Electrophoretic Mobility Shift Assay—Nuclear extracts were obtained using an extraction kit from Keygene. Equal protein amounts were incubated with the corresponding biotin-labeled DNA probes using the Chemiluminescent Nucleic Acid Detection Module (Pierce). The gel was transferred to a charged nylon membrane (Millipore) before developing. For supershift experiments, antibodies were added to the reaction mixtures. Probes were *Esrrb-P1* (forward, 5′-attgtccagccaaggccattgtcctgc; reverse, 5′-gcaggacaatggccttggctggacaat), *Esrrb-P2* (forward, 5′-caggagttcaagggcagcttgtt; reverse, 5′-aacaagctgcccttgaactcctg), and *Nanog* (forward, 5′-gagccatcctggcccattcaagggttg; reverse, 5′-caacccttgaatgggccaggatggctc).

Immunofluorescence—Cells were fixed in 4% paraformaldehyde, washed with phosphate-buffered saline, and blocked for 60 min with 10% fetal bovine serum in phosphate-buffered saline containing 0.1% Triton X-100. Secondary antibody goat anti-mouse IgM TRITC was purchased from Southern Biotech. All antibodies were diluted in 2% fetal bovine serum in phosphate-buffered saline; cells were washed with phosphate-buffered saline and mounted on 80% glycerol. A confocal microscope (LEICA TCS SP2 AOBS) was used for visualization.

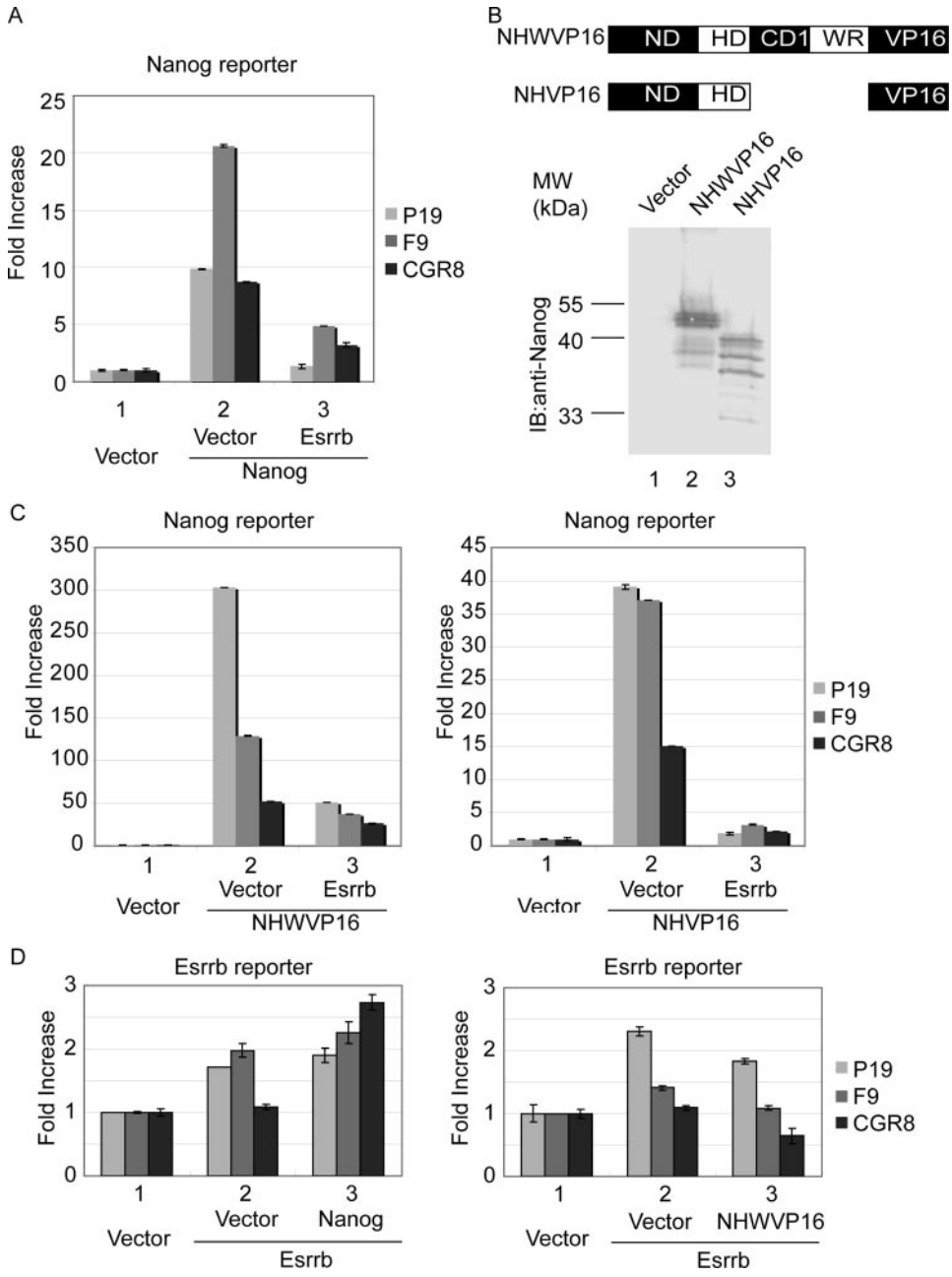

FIGURE 3. **ESRRB and NANOG reciprocally modulate their transactivation ability in pluripotent cells.** *A*, pluripotent mouse CGR8 ES cells and P19 and F9 mouse embryonic carcinoma cell lines were co-transfected with a NANOG luciferase reporter and either empty vector or *Nanog*. *Esrrb* or empty vector was also co-transfected with *Nanog*. One representative experiment of three is shown. *B*, scheme showing the domains contained in the chimeric NANOG NHWVP16 and NHVP16 proteins. Western blot of transiently transfected HEK293T cells demonstrates adequate expression and respective molecular weights of NHWVP16 and NHVP16. One representative experiment is shown. *C*, pluripotent CGR8, P19, and F9 cells were co-transfected with NANOG luciferase reporter and empty vector or NHWP16 and NHVP16; *Esrrb* or empty vector were also co-transfected as indicated. One representative experiment of three is shown. *D*, *Esrrb* and either empty vector or *Nanog* were co-transfected with ESRRB luciferase reporter in pluripotent CGR8, P19, and F9 cells. The same cell lines were co-transfected with the ESRRB reporter and either empty vector or *Esrrb*; NHWVP16 or empty vector was also co-transfected with *Esrrb* (*right*). One representative experiment of three is shown.

Real Time Reverse Transcription-PCR—RNA extraction was performed using TRIzol (Invitrogen). Equal amounts of RNA were retrotranscribed using RTAce (Toyobo), and real time reverse transcription-PCR experiments were performed using SYBR Green (Takara) and an ABI machine. Samples were normalized on the basis of β-actin values. Primers used for this study are available upon request.

RESULTS

ESRRB and NANOG Interact through Their DNA Binding Domains—Previously, immunoprecipitation and subsequent mass spectrophotometry analysis has identified ESRRB as a putative NANOG-interacting protein (10). We verified this interaction in mouse CGR8 ES cells by immunoprecipitation with anti-NANOG or anti-ESRRB and Western blotting (Fig. 1). Next, we mapped the domains involved in this interaction. Deletion mutants of NANOG and ESRRB were overexpressed in HEK293T cells, which do not express endogenous ES cell markers (*e.g. Nanog* or *Oct4*). Deletions N1, N3, N4, N5, N6, and FG-CD of NANOG; and deletions E1–E5 of ESRRB are represented in Fig. 2, *A* and *B*. NANOG N1, N3, N4, and FG-CD have been described by us before (18); FG-CD is fused to the Gal4 DNA binding domain and a FLAG tag. NANOG N5 and N6 have an amino-terminal green fluorescent protein and a Myc tag. ESRRB full-length is fused to either Myc or FLAG. All ESRRB deletions are fused to an amino-terminal FLAG tag. Western blotting with anti-NANOG, anti-Myc, or anti-FLAG antibodies showed efficient expression of these constructs (Fig. 2, *A* and *B*). Full-length NANOG (N) was co-immunoprecipitated with full length ESRRB-FLAG (E), and the interaction was maintained with ESRRB deletions E1 and E5 but not with deletions E2–E4, which lack all or part of the DNA binding domain (Fig. 2*C*). NANOG full-length and deletions N1, N2, N3, and N6, but not N5 (lacks DNA binding domain), co-immunoprecipitated with ESRRB-FLAG (Fig. 2, *D* and *E*). Immunoprecipitation with anti-FLAG antibodies of cells overexpressing NANOG FG-CD and Myc-ESRRB (Myc-E) demonstrated that ESRRB does not interact with the carboxyl-terminal domain of NANOG (Fig. 2*F*). Taken together, our results indicate that the interaction between ESRRB and NANOG is mediated through their respective DNA binding domains.

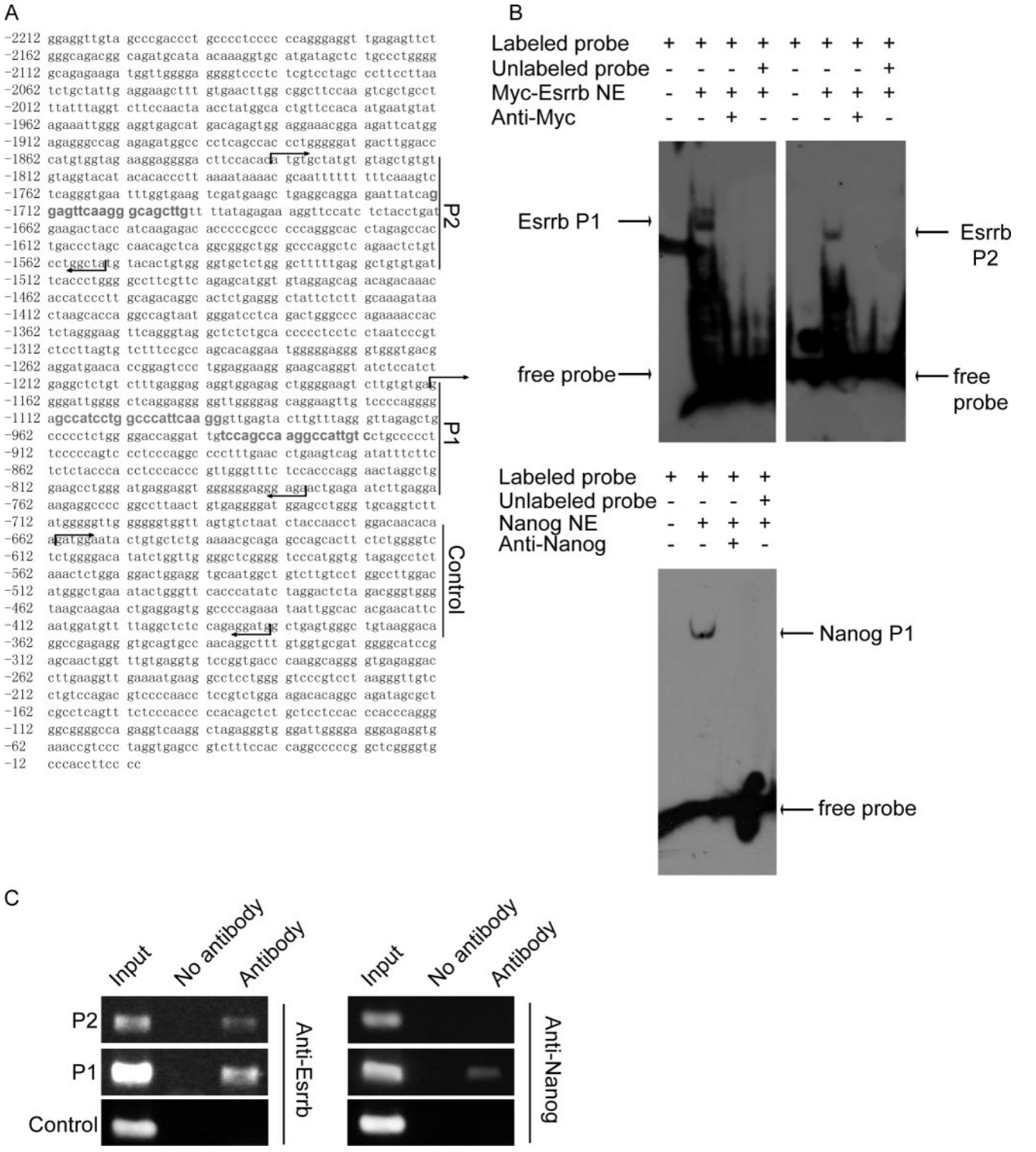

FIGURE 4. **Identification of DNA binding sites for ESRRB in the mouse *Oct4* promoter.** *A*, two potential ESRRB DNA binding sites (ESRRB P1 and ESRRB P2) and a NANOG binding site were identified in the mouse *Oct4* proximal (ESRRB P1 −921 to −939 bp, NANOG P1 −990 to −1010 bp, and ESRRB P2 −1594 to −1612) promoter using the MAT-INSPECTOR program. Both ESRRB binding sites are highlighted in *red*, and the NANOG binding site is highlighted in *green*. *B*, a short DNA probe containing the ESRRB-P1, ESRRB-P2, or NANOG binding sites was biotin-labeled and incubated with nuclear extracts (NE) from HEK293T cells transiently transfected with Myc-*Esrrb* or *Nanog*. Samples were run on an acrylamide gel. Labeled probe without NE, and excess unlabeled probe incubated with labeled probe (for specific competition) plus NE were used as controls. Anti-Myc or anti-NANOG antibodies demonstrated that the bands are specific; no supershift was detected. One representative experiment of three is shown. *C*, ChIP with anti-ESRRB or anti-NANOG antibodies of CGR8 cells stably overexpressing *Nanog*-FLAG or Myc-*Esrrb*; similar results were obtained with anti-Myc and anti-FLAG antibodies (data not shown). Semiquantitative PCR was performed with specific oligonucleotides that amplify the indicated DNA fragments. One representative experiment of three is shown.

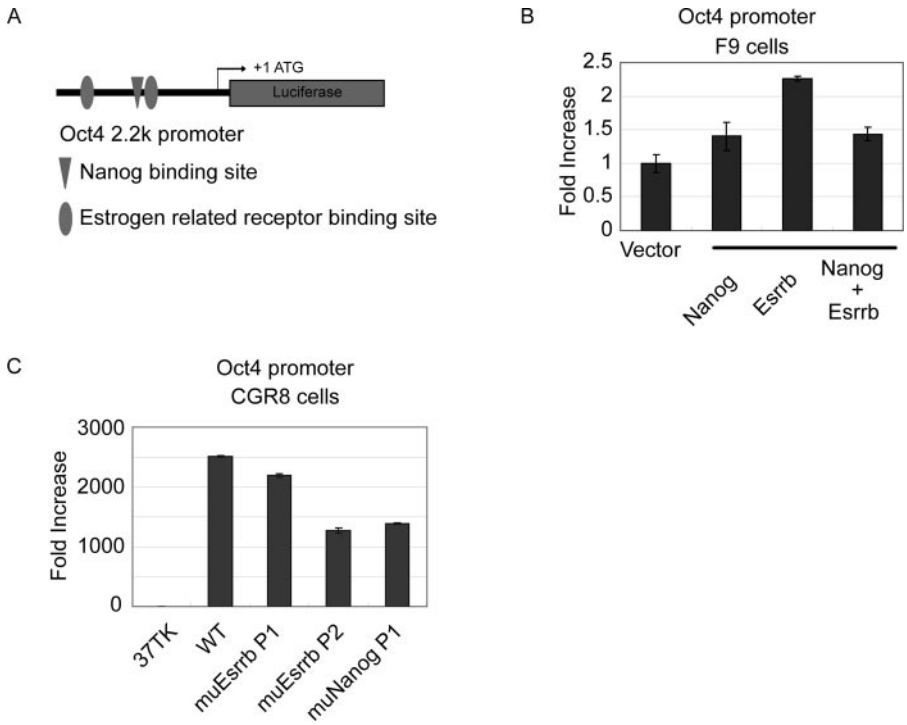

FIGURE 5. **ESRRB and NANOG activate transcription of the *Oct4* promoter.** *A*, scheme of the luciferase reporter plasmid containing the *Oct4* proximal promoter described in Fig. 4*A*. *B*, effect of transiently co-transfecting *Nanog* or *Esrrb* with the *Oct4* promoter reporter in pluripotent F9 cells. One representative experiment of three is shown. *C*, effect of mutating ESRRB or NANOG binding sites in the basal *Oct4* promoter reporter activity in transiently transfected CGR8 cells. Mutation of ESRRB-P2 or NANOG binding sites potently reduced ESRRB and NANOG-mediated transactivation of the *Oct4* promoter reporter in transiently transfected HEK293T cells (data not shown). One representative experiment of three is shown.

ESRRB and NANOG Reciprocally Modulate Their Transactivation Ability in Pluripotent Cells—NANOG was originally described as a transcriptional repressor (23, 24). We have previously reported that it can also act as a potent transactivator (18), thanks to two unusually potent transactivation domains (WR and CD2) located in the carboxyl-terminal edge (see Fig. 2*A*). We thus hypothesized that the interaction between ESRRB and NANOG might result in increased transcriptional activity of the latter, since this could potentially explain why *Esrrb* knockdown induces ES cell differentiation. Paradoxically, forced expression of *Esrrb* in CGR8 and mouse embryonic carcinoma cell lines (P19 and F9) repressed the activation mediated by NANOG of a reporter gene containing NANOG binding sites (Fig. 3*A*). A similar experiment was performed using chimeric proteins of NANOG that lack either the CD2 terminal domain (NHWVP16) or the entire carboxyl-terminal transactivation domain (CD1, WR, and CD2) (NHVP16) and are fused to the viral VP16 transactivation domain (see scheme in Fig. 3*B*). Western blotting showed potent expression of both NANOG fusion proteins in HEK293T cells (Fig. 3*B*). ESRRB reduced the activity of both NHWVP16 and NHVP16 in pluripotent cells (Fig. 3*C*), demonstrating that the repressive effect does not involve the carboxyl-terminal transactivation domain of NANOG.

ESRRB can bind to specific DNA response elements and activate transcription (*e.g.* in the case of the pS2 gene) (22). We evaluated whether NANOG could influence ESRRB transactivation ability in this setting. Forced expression of NANOG moderately induced ESRRB mediated-transcriptional activity in P19 and F9 cells and more potently in CGR8 ES cells (Fig. 3*D*). This synergistic effect was more dependent on the CD2 domain of NANOG than the WR, since it was abolished when NHWVP16 (which only lacks CD2) was overexpressed together with ESRRB and the pS2 reporter (Fig. 3*D*). Hence, the interaction between ESRRB and NANOG has the potential to reciprocally modulate the transcriptional activity of both.

ESRRB and NANOG Independently Activate Oct4 Transcription—Given the above results, we speculated that ESRRB might be controlling ES cell behavior by binding to and activating the promoters of key ES cell core factors. We chose *Oct4* as a putative target because tight control of *Oct4* levels is essential to prevent ES cell differentiation (12). Using the MAT-INSPECTOR program (Genomatix), we identified two degenerate ESRRB DNA binding sites (ESRRB-P2 and ESRRB-P1) (25) in the mouse proximal *Oct4* promoter (Fig. 4*A*). ESRRB-P1 is located near a putative NANOG site. Specific binding of ESRRB or NANOG to these sequences was demonstrated by electrophoretic mobility shift assay with labeled oligonucleotide sequences that comprise either ESRRB-P2 or ESRRB-P1 or the NANOG binding site. Nuclear extracts were prepared from HEK293T cells transiently transfected with Myc-*Esrrb* or *Nanog*. ESRRB bound to both ESRRB-P1 and ESRRB-P2, and NANOG bound to its putative cognate sequence (Fig. 4*B*). Binding was effectively eliminated in both cases when anti-Myc or anti-NANOG antibodies were added to the reaction mixture (Fig. 4*B*). ChIP analysis of CGR8 cells stably expressing Myc-*Esrrb* (to increase efficiency of the capture) further demonstrated that both ESRRB-P2 and P1 are *bona fide* ESRRB binding sites (Fig. 4*C*). Only the DNA fragment containing the NANOG binding site could be immunoprecipitated with anti-NANOG antibodies in CGR8 cells stably expressing NANOG-Flag (Fig. 4*C*). We next used a luciferase reporter plasmid containing the *Oct4* promoter described in Fig. 4*A* (see the scheme in Fig. 5*A*). Overexpression of *Nanog* and *Esrrb* activated the *Oct4* promoter reporter in F9 when transfected individually, but co-transfection did not result in synergy but rather in antagonism (Fig. 5*B*). This effect could be related to the above described interplay between ESRRB and NANOG. Overexpression of either transcription factor in CGR8 cells could only activate the *Oct4* reporter modestly (data not shown), which is probably due to high levels of endogenous *Esrrb* and *Nanog*. We therefore mutated

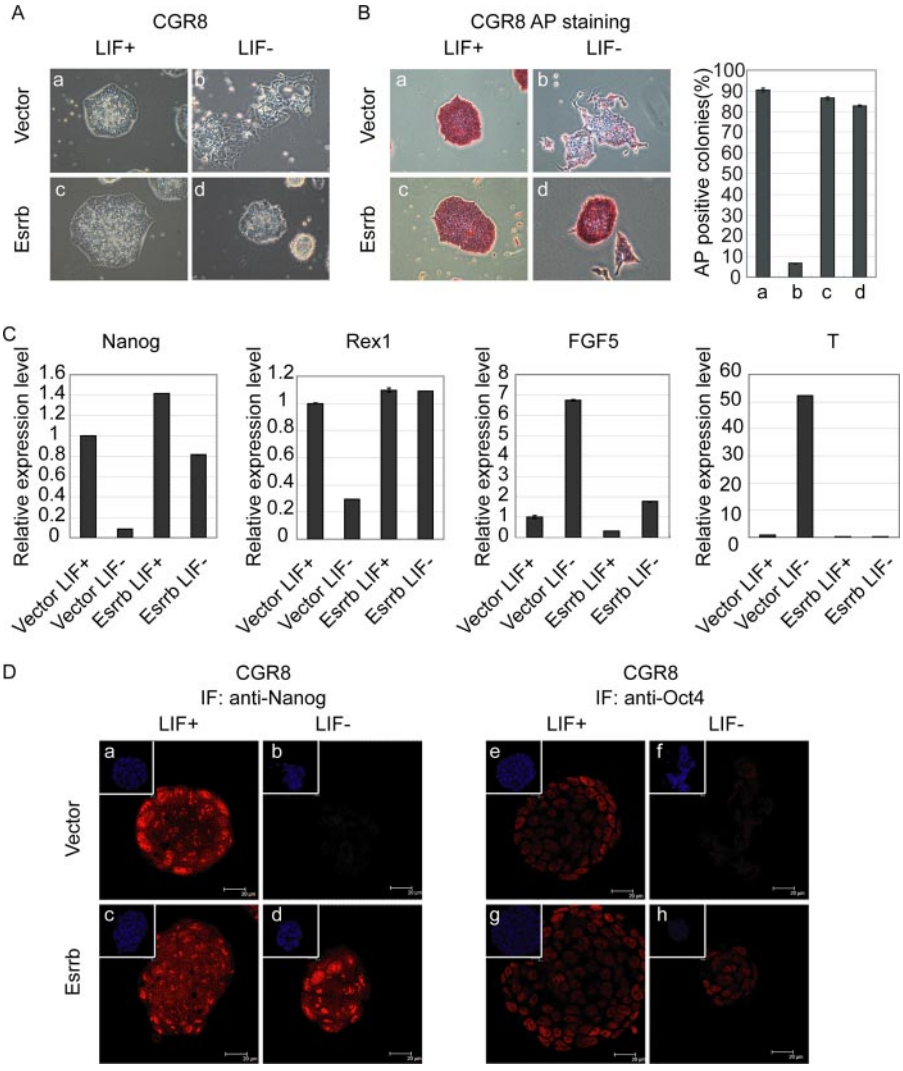

FIGURE 6. ***Esrrb* is sufficient to maintain ES pluripotency and self-renewal in the absence of LIF.** *A*, CGR8 cell lines stably transfected with empty vector or Myc-*Esrrb* were cultured in the presence of LIF or deprived of LIF for 6 days, after which phase-contrast pictures were taken. Most if not all colonies remained undifferentiated in *Esrrb*-overexpressing LIF cultured in either condition. This behavior was reproduced in multiple experiments. *B*, representative AP staining of the same cell lines with or without LIF. Quantification is shown in the *right panel*. This pattern was maintained in multiple experiments. *C*, real time reverse transcription-PCR of selected genes associated with ES cell pluripotency or differentiation. One representative experiment of three is shown. *D*, immunofluorescence microscopy (*IF*) with anti-NANOG and anti-OCT4 of the same CGR8 cell lines. Representative fields are shown. The experiment was repeated three times.

ESRRB-P1 and ESRRB-P2 or the NANOG binding site; the latter 2 mutations had a potent effect in reducing the basal activity of the *Oct4* promoter in CGR8 cells (Fig. 5*C*). In conclusion, ESRRB and NANOG independently bind to and activate *Oct4* transcription.

Esrrb Can Sustain ES Cell Pluripotency and Self-renewal in the Absence of LIF—To investigate whether *Esrrb* alone is sufficient to maintain ES cell characteristics, we stably transfected CGR8 (Fig. 6) and E14T cells (Fig. 7) with either empty vector or *Esrrb*. The resulting cell lines were then cultured in the presence or absence of LIF for 6 days, after which cell morphology and AP activity were evaluated (Figs. 6, *A* and *B*, and 7, *A* and *B*). AP staining was high in *Esrrb*-overexpressing ES cells cultured without LIF and faint or absent in the control. *Esrrb*-overexpressing colonies also retained the classic compact morphology with well defined borders of dedifferentiated ES cell colonies, whereas control cells were flat and displayed abundant cytoplasmic prolongations. Real time reverse transcription-PCR analysis of ES cell marker genes, including *Nanog* and *Rex1*, demonstrated comparable levels in *Esrrb*-stable ES cell lines cultured with or without LIF (Figs. 6*C* and 7*C*). LIF depletion in *Esrrb*-stable ES cells did not influence mRNA levels of genes (Fgf5 and T) known to increase after ES cell differentiation (Figs. 6*C* and 7*C*). In addition, immunofluorescence staining for NANOG and OCT4 showed homogeneous high levels in ESRRB-overexpressing CGR8 cells cultured without LIF and low in the control (Fig. 6*D*).

DISCUSSION

The list of proteins involved in controlling ES cell behavior is growing steadily. In many cases, like it happens with ESRRB, it is poorly understood how they exert their roles. ESRRB has been reported to interact with the ES cell master regulator NANOG (10). Multiple other proteins also interact with NANOG, and some of them are also transcription factors. It is, for example, the case of Dax1, Sall4, and NF-κB (10, 26, 27). Small interfering RNA for Dax1 or Sall4 induces differentiation of ES cells (28, 29). Dax1 belongs, like *Esrrb*, to the family of orphan nuclear receptors and has a known role in the establishment and maintenance of steroid-producing tissues like the testis and the adrenal gland (30). How Dax1 helps control pluripotency is probably unrelated to its role in steroidogenesis. Sall4 is a member of the Spalt-like family of proteins; Sall4 co-occupies with NANOG the promoters of many genes and is needed for NANOG-mediated transactivation of these targets (26). High levels of NF-κB induce differentiation of ES cells by activating lineage-specific programs. NF-κB binds to the WR transactivation domain of NANOG, and this results in repression of the transcriptional activity of the latter (27).

ESRRB can bind to specific DNA recognition sequences and is transcriptionally active in the absence of exogenously added ligand. Two ESRRB binding sites were identified in the proximal mouse *Oct4* promoter, one of which is responsible for maintaining *Oct4* transcription together with NANOG. The positive effect of ESRRB on *Oct4* may on its own explain

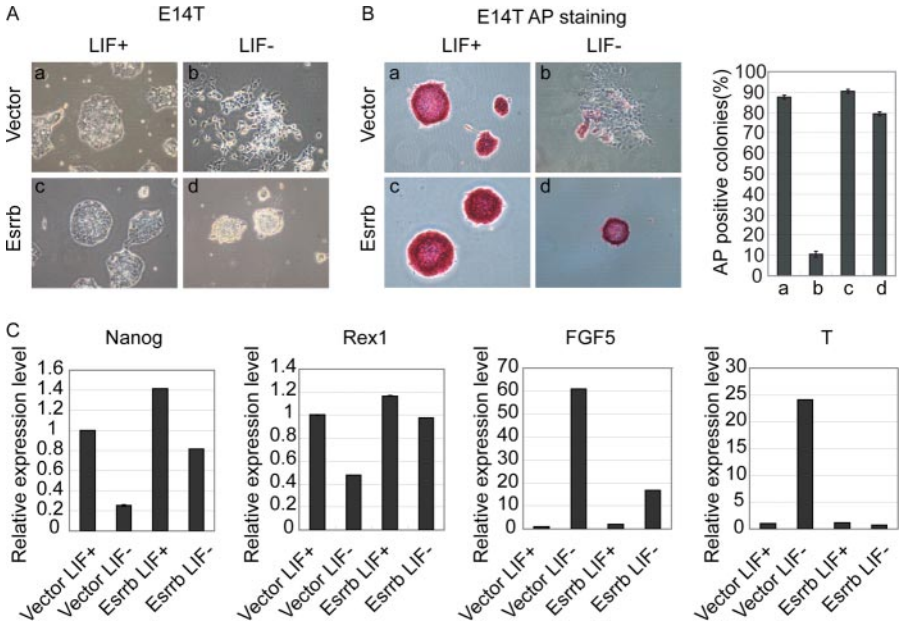

FIGURE 7. ***Esrrb* is sufficient to maintain ES pluripotency and self-renewal in the absence of LIF.** *A*, E14T cell lines stably transfected with empty vector or Myc-*Esrrb* were cultured in the presence of LIF or deprived of LIF for 6 days, after which phase-contrast pictures were taken. Most if not all colonies remained undifferentiated in *Esrrb*-overexpressing LIF cultured in either condition. This behavior was reproduced in multiple experiments. *B*, representative AP staining of the same cell lines with or without LIF. Quantification is shown in the *right panel*. This pattern was maintained in multiple experiments. *C*, real time reverse transcription-PCR of selected genes associated with ES cell pluripotency or differentiation. One representative experiment of three is shown.

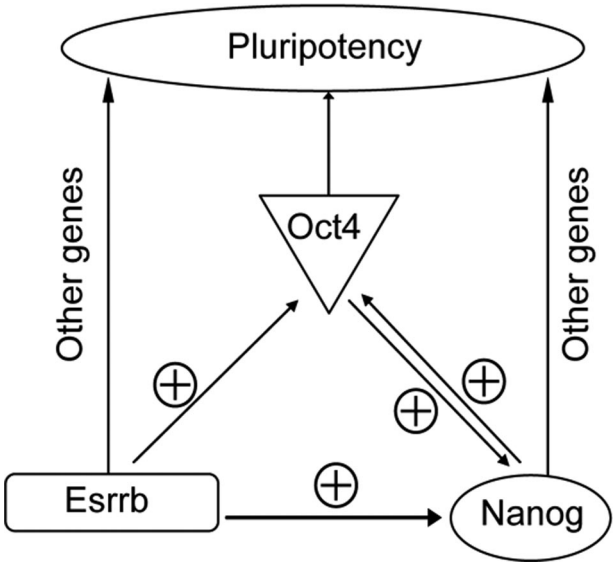

FIGURE 8. **Scheme of how the interplay between *Esrrb*, *Nanog*, and *Oct4* helps control pluripotency in ES cells.** This figure summarizes the results presented in this paper and also the data reported by others (31, 32).

why small interfering RNA for *Esrrb* induces differentiation. Further supporting this idea, we have demonstrated that overexpression of *Esrrb* alone is able to maintain self-renewal and pluripotency in ES cells in the absence of LIF (see the scheme in Fig. 8). While our manuscript was under review, ChIP-on-chip analysis in mouse ES cells demonstrated the existence of ESRRB binding sites through the whole genome (31). Many of these potential ESRRB target genes comprise OCT4, SOX2, and NANOG-regulated genes and include the respective promoters of these core ES cell regulators too. Also, van den Berg *et al.* (32) demonstrated that ESRRB binds to *Nanog*, *Rest*, and *Rex1* promoters. For the former two promoters, binding of ESRRB to its cognate sequence required *Oct4* being bound to a nearby site. This was, however, not needed for ESRRB binding to the *Rex1* promoter. Paradoxically, ESRRB was capable of transactivating *Nanog* and *Rex1* promoters but not *Rest*. In the context of our study, ESRRB could repress the ability of NANOG to transactivate a NANOG reporter gene, whereas NANOG had the opposite effect on ESRRB. This suggests that the interplay between both factors may reciprocally affect their ability to activate common targets and thus fine-tune ES cell pluripotency. But this interplay is likely to be target-specific and complex. Likewise, it is conceivable that the number of ESRRB-interacting proteins in ES cells will extend beyond OCT4 and NANOG in the same way that most ES cell transcription factors interact with and cross-regulate each other. Future work will be important to evaluate these considerations.

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Esrrb Activates *Oct4* Transcription and Sustains Self-renewal and Pluripotency in Embryonic Stem Cells

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